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13. ABSTRACT ***(Maximum 200 Words)***

Tumor growth, progression, and metastasis are dependent on the ability of the tumor to recruit a blood supply. It is currently believed that tumors become vascularized by a process referred to as angiogenesis. We propose that a second process of blood vessel formation, vasculogenesis, a process not currently associated with tumor vascularization, is operative in breast cancer. Although it is well established that all new blood vessels are formed by either angiogenesis or vasculogenesis, it is believed that only angiogenesis, the formation of new blood vessels from existing blood vessels, occurs in adults. Recent evidence suggests that this may not be the case and that vasculogenesis, a process currently associated with the formation of blood vessels in the embryo, is operative in adults. That vascularization is supported by experiments that suggest that mesodermally derived stem cells are found at sites where new blood vessels are forming in adults. If we are correct that the vessels of the rumor are in part or fully formed by vasculogenesisi, then our knowledge of this process, which has mechanisms that are distinct from angiogenesis could be used to combat the progression of the breast tumor.

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INTRODUCTION

The Role of Vasculogenesis in Breast Cancer

Tumor growth, progression, and metastasis are dependent on the ability of the tumor to recruit a blood supply. It is currently believed that tumors become vascularized by a process referred to as angiogenesis (Folkman 1971). We propose that a second process of blood vessel formation, vasculogenesis, a process not currently associated with tumor vascularization, is operative in breast cancer (Risau and Flamme 1995).

The specific aims of the grant are:

AIM 1. To determine if the bone marrow is a source of precursor cells participating in adult vasculogenesis experiments will be conducted to determine:

a) if the bone marrow is the source of endothelial cells participating in new blood vessel formation

b) in what compartment (bone marrow, peripheral blood, or at the site of vessel assembly) are endothelial cell precursors/angioblasts first detected

AIM 2. To determine the relevance of vasculogenesis to breast cancer experiments will be conducted to determine:

a) the source of endothelial cells that form new blood vessels in breast tumors using nude mice

b) the source of endothelial cells that form new blood vessels in breast tumors using a transgenic mouse model of breast cancer

AIM 3. To develop and characterize a vasculogenic assay for use in screening potential anti-vasculogenic/anti-tumor reagents experiments will be conducted to determine:

a) the morphogenic fidelity of in vitro vasculogenesis

b) if the response of endothelial progenitors/endothelial cells in the assay recapitulates the response of similar cells in vivo

BODY

STATEMENT OF WORK IN YEAR 1: progress on tasks with a time frame of 1-12 months (Tasks 1-4, and 8). These tasks are embodied in Aims 1 and 3 as follows: Aim 1 Tasks 1 and 4 and Aim 3 Tasks 2, 3 and 8.

AIM 1 *Task 1.* To determine if the bone marrow is a source of precursor cells participating in adult vasculogenesis we will generate chimeric mice that will allow us to trace endothelial precursor cells that are derived from the bone marrow (months 1-6).

The accomplishment of Aims 1 and 2 requires the development of chimeric mice with "tagged bone marrow". The generation of such mice allows the contribution of adult vasculogenesis from bone derived stem cells to be evaluated in both normal development and as part of blood vessel formation associated with breast tumor development. As outlined in the application a number of approaches were envisioned for the generation of such animals. Examples are the development of "green" chimeric mice in which Green Fluorescent Protein (GFP) is expressed under the endothelial specific promoter Tie2, or the development of "blue" chimeric mice from the Rosa 26 animals (Percival and Slack 1999; Suri et al., 1996; Schlaeger et al., 1997; Abrahamson et al., 1998).

Through our collaboration with Dr. Makio Ogawa (Department of Medicine MUSC), we are in the process of generating chimeric mice necessary for Aims 1 and 2. Dr. Ogawa's access to the GFP mouse, a mouse in which all cells express green florescent protein (Hadjantonakis et al., 1998), has allowed us to make progress in developing the tagged mouse necessary for the accomplish of Aims 1 and 2. By transplanting cells derived from peripheral blood or from the bone marrow of GFP animals into normal mice that have had their bone marrow cells eradicated , we hope to generate "normal mice" with GFP labeled bone marrow. To date we have evaluated the bone marrow of these chimeric mice and have shown that the GFP cells have successfully engrafted. Analysis of the contribution of the engrafted cells to adult vasculogensis is anticipated to begin within two months. Aim 1 Task 4 (Months 6-12) to determine the compartment (bone marrow, peripheral blood, or at the site of vessel assembly) in which precursors of endothelial cells, angioblasts, are generated is contingent on progress in Aim 1Task 1 Work on this task is expected to begin within 6 months.

Negative results - there have been no results that would suggest that the Aims as stated in the application are not achievable, or that the original hypotheses are not valid.

Problems - no major problems

Recommended changes to better address the work - the use of the GFP mouse to generate mice with labeled bone marrow was not included in the original research plan. Based on the positive preliminary data suggesting that we have been successful in generating the chimeric mice with tagged bone marrow (GFP labeled), we will focus on the development of this model. The successful

development of this model fulfills all of the research needs that the original approaches would have achieved.

AIM 3, *Tasks 2, 3 and 8*

Task 2. To develop and characterize a vasculogenic assay for use in screening potential anti-vasculogenic/anti-tumor reagents. (Months 1-36)

- characterization of the vasculogenesis assay (months 1-12).

Task 3. To develop and characterize a vasculogenic assay for use in screening potential anti-vasculogenic/anti-tumor reagents (Months 1-36)

- to test the ability of our vasculogenesis assay to mimic previously described in vivo responses

Task 8. To develop and characterize a vasculogenic assay for use in screening potential anti-vasculogenic/anti-tumor reagents (Months 1-36)

- to use this assay to evaluate reagents for anti-vasculogenic activity

The overall objective of the tasks in Aim 3 are the development and characterization of an in vitro assay of vasculogenesis for use in screening potential anti-vasculogenic/anti-tumor reagents. The work related to this Aim as described below are in part embodied in a manuscript that is in preparation and will be submitted within a month (see attached).

A novel *in vitro* assay of vasculogenesis

Essential to elucidating the mechanisms regulating endothelial cell behaviors associated with both normal and pathological neovascular processes are in vitro models. Indeed the rapid advances made in understanding angiogenesis have been due in part to the availability of such models. Unfortunately, to date there are a limited number of in vitro assays of vasculogenesis (Doetschman et al., 1985; Hatzopoulos et al., 1998). The difficulty in generating vasculogenic models is that they require a source of undifferentiated mesodermal cells. As part of this application we sought to develop an in vitro assay of vasculogenesis using the murine allantois. The fact that the mouse allantois represents a pure population of the very cells (splanchnic mesodermal cells) that give rise to the endothelial cell lineage makes it ideally suited for the development of an assay (Downs 1998; Downs et al., 1998; Drake and Fleming 2000). At the time of submission we presented preliminary data demonstrating that explanted prevascular allantoides form blood vessels de novo by the process of vasculogenesis.

Efforts during the past year have furthered our biochemical and morphological understanding of allantoic vasculogenesis and most importantly have demonstrate the model to be a faithful recapitulation of vasculogenesis as it occurs *in vivo*. Additional research during the first year has

lead to the development of a new component to the assay that provides a means of quantitatively measuring the dynamics of vasculogenesis occurring both *in vivo* and *in vitro*.

Biochemical and morphological characterization of allantoic vasculogenesis demonstrates: 1) that the temporal expression of the proteins TAL1, Flk1, PECAM (CD31), CD34 and VE-cadherin is identical to that observed in the embryo, 2) the presence of numerous angioblasts ($TAL1^+/Flk1^+$), but a lack of blood vessels and/or endothelial cells ($TAL1^+/Flk1^+/CD31^+$) in the 6.5-7.5 dpc allantoides, as judge by immunolabeling studies using antibodies to TAL1, Flk1 and CD31, 3) that when cultured for 12-18 hrs., pre-vascularized allantoides of 6.5-7.5 dpc mouse embryos form networks of blood vessels, and 4) that the blood vessels formed in vitro form lumens, the defining hallmark of blood vessels.

Vasculogenesis *in vitro* is a faithful recapitulation of the process as it occurs *in vivo*. To determine if this model faithfully recapitulated vasculogenesis *in vivo,* we compared the response of the model to VEGF to the previously reported in vivo response to VEGF. Treatment of either 7.5 or 8.5 dpc allantois explants with exogenous VEGF transformed the delicate vascular networks observed in controls into vascular sinuses, a response which was similar to that observed in embryos injected with VEGF (Drake and Little 1995). Additionally, when thin sections of the culture were analyzed, individual vessels characteristic of the normal culture were replaced by vascular sinuses resembling those generated in avian embryos in response to VEGF (Drake and Little 1995). The fact that vasculogenesis *in vitro* shared morphological, biochemical and functional properties with the process as it occurs *in vivo* led us to conclude that the *in vitro* process was a faithful recapitulation of the process as it occurs *in vivo*.

VEGF elicits differing responses depending on the cell type, angioblasts versus endothelial cells. Having established that the *in vivo* and *in vitro* response of the model to VEGF A were identical we next evaluated whether angiobalst and endothelial cells respond similarly when VEGF/VEGFR signaling is perturbed. When either embryos or cultured 7.5 dpc allantoides were evaluated for the effects of a soluble form of the VEGF receptor Flt (sFlt1) or antibodies to VEGF the response was the same, the abrogation of vascular network formation. In contrast, the treatment of cultured pre-vascularized 8.5 dpc allantoides or embryos with an established vasculature with either sFlt1 or antibodies to VEGF revealed no apparent changes in blood vessels morphogenesis.

The cellular dynamics of vasculogenesis as revealed by fluorescence-activated cell scanning analysis. We have employed fluorescence-activated cell scanning (FACS) as a means of quantitatively measuring the dynamics of vasculogenesis occurring both *in vivo* and *in vitro*.

Specifically, we can measure numbers of mesodermal cells, angioblasts and endothelial cells throughout the process of vasculogenesis. Such cells are defined by expression of specific cell surface markers that we and others have described (Yamaguchi et al., 1993; Baldwin et al., 1994; Drake and Little 1995; Downs et al., 1998). Using this approach we have defined the following cell populations as participants in vasculogenesis: primitive mesodermal cells ($Flk1^-/CD31^-$), angioblasts ($Flk1^+/CD31^-$), and endothelial cells ($Flk1^+/CD31^+$). We have created FACS profiles for VEGF in the allantois culture. VEGF treatment produced a 2-fold increase in the *percentage* of endothelial cells and a 2-fold increase in *total cell numbers* for a total of a 4X increase in endothelial cells numbers. Our preliminary FACS data and FACS analysis by others using embryonic stem cells support the concept that the mesoderm has a gradient of $Flk1^+$ cells. We believe that this gradient represents a spectrum of cell potentials ranging from pluripotent mesoderm to angioblasts.

Negative results - there have been no results that would suggest that the Aims as stated in the application are not achievable, nor that the original hypotheses are not valid

Problems - no major problems

Recommended changes to better address the work - none

KEY RESEARCH ACCOMPLISHMENTS

- The characterization of biochemical and morphological properties of allantoic vasculogenesis
- The demonstration that vasculogenesis *in vitro* is a faithful recapitulation of the process as it occurs *in vivo*
- The finding that VEGF elicits differing responses depending on the cell type, angioblasts versus endothelial cells
- The cellular dynamics of vasculogenesis as revealed by fluorescence-activated cell scanning analysis allows quantitative analysis of the process
- Development of animal models for the analysis of adult vasculogenesis from bone marrow derived stem cells

REPORTABLE OUTCOME

See attached manuscript

CONCLUSIONS: IMPORTANCE AND IMPLICATIONS

Progress in development of the mouse model necessary for Aims 1 and 2 was made in the first year with the development of the GFP chimeric mice. Mice having tagged bone marrow will provide the research tool needed to investigate the role of vasculogenesis from circulating cells in breast cancer. As stated in the original application the identification of a vasculogenic component to the formation of blood vessels in breast tumors would have profound implications as regards potential treatments. The development of drugs that target vasculogenesis is the subject of Aim 3. This Aim has moved most quickly and is the subject of a manuscript in preparation. The most important result to date using this assay is that angioblasts, the cells that drive vasculogenesis, are far more susceptible to certain anti-neovascular reagents then are the endothelial cells of established vessels. That these cells respond differently strongly supports the overall hypothesis of the grant.

SO WHAT SECTION

If blood vessels associated with tumor vascularization in breast cancer are generated as a result of both angiogenesis and vasculogenesis as proposed in this application, then the establishment of this fact will lead to a reconsideration of current anti-neovascular strategies. That this would be the case is indicated by our findings that cells associated with these two neovascular processes respond differently to anti-neovascular reagents, with vasculogenic associated angioblasts being more susceptible then the endothelial cells that are associated with angiogenesis.

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APPENDICES

Allantois explant culture: A novel *in vitro* assay of vasculogenesis

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Abstract

Herein, we describe a novel *in vitro* model of vasculogenesis, the *de novo* formation of blood vessels. We show that blood vessels formed in cultured pre-vascularized mouse allantois explants are morphologically similar to those formed *in vivo*. Furthermore, proteins characteristic of *in vivo* vasculogenesis are appropriately expressed during *in vitro* vasculogenesis. Using this model, we have tested the effects of VEGF, VEGF antibodies and a soluble form of the VEGF receptor, Flt1. VEGF treatment resulted in the formation of hyperfused blood vessels similar to those observed in embryos injected with VEGF or in transgenic mice over-expressing VEGF. In contrast, VEGF antibodies and soluble Flt1 abrogated the assembly of blood vessels in allantois explants, as each did *in vivo*. Therefore, the *in vitro* model mimics *in vivo* morphogenic and biochemical aspects of vasculogenesis and responds appropriately to vasculogenic effectors. The attributes of this system afford new opportunities to understand vasculogenesis and identify drugs that modulate the process.

Introduction

Vasculogenesis is the *de novo* formation of blood vessels from mesodermal precursor cells. It has become evident that this process, once thought to be operative exclusively in embryos, contributes to blood vessel formation in adults[1-7]. This realization is capturing the attention of researchers interested in developing new therapies for controlling neovascularization. Advancement in the understanding of vasculogenesis will benefit from *in vitro* models, as was the case for angiogenesis research[8]. Currently, the most widely used model of vasculogenesis employs embryonic stem cells in embryoid body culture[9-11]. While this system recapitulates many aspects of *in vivo* vasculogenesis, the concurrent formation of diverse vascular morphologies (e.g., primary vascular networks and sinusoidal vessels) in a single embryoid body represents a shortcoming. Ideally, a system in which vascular morphogenesis proceeds in coordinated manner, displaying a singular transition from undifferentiated mesoderm to networks of blood vessels and then sinuses, would be preferred. Herein we define an *in vitro* model embodying these and other desirable features.

Results

Vascular morphogenesis in the developing allantois

The murine allantois is an outcropping of extra-embryonic splanchnic mesoderm that participates in the formation of the chorioallantoic placenta. The first blood vessels that arise in the allantois are generated by vasculogenesis[12,13]. Using confocal microscopy we show that there are no discernable blood vessels ($PECAM^+$ cells) in the 7.5 dpc allantois (Fig. 1A). By 8.5 dpc, a $PECAM^+$ central vessel was evident extending along the length of the allantois surrounded by a dense vascular network (Fig. 1B).

The early allantois contains endothelial progenitors

Co-expression of TAL1 and Flk1 has been associated with angioblasts in the embryo proper[13]. In pre-vascularized 6.5-7.5 dpc allantoides, isolated cells expressing both proteins were detected (Fig. 2). TAL1 staining was localized to the nucleus and in cytosolic aggregates while Flk1 staining was diffusely distributed throughout the cytosol and in peri-nuclear aggregates (Fig. 2A, B, C). $TAL1^+$ cells of the 6.5-7.5 dpc allantoides lacked detectable PECAM expression (Fig. 2D, E, F). At 8.5 dpc, angioblasts ($TAL1^+$ cells) were present in areas adjacent to $PECAM^+$ blood vessels (Fig. 2G, H).

Murine allantois cultures recapitulate aspects of in vivo vasculogenesis

Ex vivo culture of murine embryonic allantoides has been described previously[12,13]. As shown in Figure 3, when pre-vascularized 7.5 dpc allantoides were cultured blood vessels formed *de novo*. Furthermore, the constituent cells of the newly formed vessels express the endothelial cell markers Flk1, CD34 and VE-cadherin (Fig. 3). In addition, isolated $Flk1^+$ cells (angioblasts) were present in avascular regions (Fig. 3B). These cells lacked detectable expression of the endothelial markers CD34, VE-cadherin (Fig. 3C and D) and PECAM (data not shown). Analysis of thin sections

verified that vessels formed *in vitro* had lumens (Fig. 4B). When examined at higher magnification zonula adherens junctions were detected (Fig. 4C). Taken together, these findings indicate that vessels formed in explant cultures have morphological and biochemical features consistent with vessels formed *in vivo*.

New blood vessel formation was also observed when vascularized (8.5 dpc) allantois explants were cultured. By comparison to the networks generated from culture of 7.5 dpc allantoides (Fig. 5A), the vascular networks of 8.5 dpc allantoides (Fig. 5B) are more complex as judged by the greater number of branch points/unit area (14.6–6.0, n=10 versus 30.0–9.7, n=10, respectively) and nodes/unit area (7.9–3.6, n=10 versus 15.9–5.2, n=10, respectively). Further, the vascular density (VD) of blood vessels in the cultured 8.5 dpc allantois is ~2 fold greater than that of the vessels in the 7.5 dpc cultured allantois (14.0–3.1, n=20 and 7.4–2.5, n=16, respectively). Given the fact that the 8.5 dpc allantoides contain angioblasts as well as established blood vessels (Fig. 2G, H), it is possible that vasculogenesis contributes to the augmented vascularization observed in the cultured 8.5 dpc allantoides.

Effectors of in vivo vasculogenesis elicit similar responses in vitro

Vasculogenesis *in vivo* can be modulated in a predictable manner by exogenously added agents. For example, microinjection of $VEGF_{165}$ into embryos transforms small caliber vessels into sinusoidal vessels (Fig. 6B), while a soluble form of VEGF receptor-1 (sFlt1) disrupts blood vessel formation (Fig. 6C)[13,14]. We therefore investigated whether the allantois explant culture would respond in a manner similar to *in vivo*. VEGF treatment of 7.5 and 8.5 dpc allantois explants resulted in profound alterations in vascular patterning, the avascular regions typically observed in control cultures were reduced and replaced by endothelial cells ($PECAM^+$) (Fig 6F, J). This response is similar to that observed in embryos injected with VEGF (Fig. 6B). Analysis of thin sections from VEGF-treated allantois explants showed that small caliber vessels characteristic of control explants had been replaced by large vascular sinuses (Fig. 6M). We next evaluated the response of the culture to treatment with sFlt1 or VEGF

antibodies. Treatment of 7.5 dpc allantoides with either agent resulted in the abrogation of vascular network formation (Fig. 6G, H). Small aggregates of $PECAM^+$ cells were observed in place of networks. These results are consistent with sFlt1- and VEGF antibody-induced alterations observed *in vivo* (Fig. 6C, D). By contrast, treatment of vascularized 8.5 dpc allantoides with sFlt1 and VEGF antibodies produced no apparent effect on vascular network formation (Fig. 6K, L).

Reproducibility observed using explant culture as an assay system

To assess the reproducibility of the response of *in vitro* vasculogenesis to treatment with exogenous agents we measured the vascular density (VD) in agent-treated explants (Table I). VD values determined from explants treated with sFlt1 deviated from the mean by <0.5% for 7.5 dpc allantoides (n=7) and by <4% for 8.5 dpc allantoides (n=8). Similarly, VD values for VEGF-treated cultures deviated from the mean by <6% for 7.5 dpc allantoides (n=6) and <1.3% for 8.5 dpc allantoides (n=5). Finally, VD values for VEGF antibody-treated cultures deviated from the mean by <0.6% for 7.5 dpc allantoides (n=3) and <2.2% for 8.5 dpc allantoides (n=4). In addition to demonstrating that VD measurements from control, sFlt1-, VEGF- and VEGF antibody-treated 7.5 dpc allantois cultures cluster, statistically significant differences (p values < 0.001) were found between VD values of controls and treated cultures. In contrast to findings from 7.5 dpc allantoides cultures, only VD values from the VEGF-treated 8.5 dpc allantois cultures were statistically different (p values $<10^{-7}$) from controls. VD values from control, sFlt1-, and VEGF antibody-treated 8.5 dpc allantois cultures were found to cluster, however, the values were not statistically different (Table I). The fact that VD values of sFlt1- and VEGF antibody-treated 8.5 dpc cultures did not differ significantly from controls (Table I) is consistent with visual assessment (Fig. 6) which indicates no apparent effect of these agents on vascular morphogenesis of 8.5 dpc allantois explants. These findings show a relatively low variability in the density of blood vessels formed during normal allantois explant culture. Furthermore, agent-induced alterations in VD are consistent for each agent and can be statistically differentiated from controls.

Discussion

Herein we introduce the allantois explant culture as a tool for studying vasculogenesis and as a system to assay the consequence of agents on the morphological and molecular aspects of the process. We show that this culture system recapitulates biochemical and morphological events of *in vivo* vasculogenesis. Specifically, primitive mesoderm of 7.5 dpc allantoides differentiates into $TAL1^+/Flk1^+$ endothelial precursor cells (angioblasts) which subsequently give rise to $TAL1^+$/ $Flk1^+$/ $PECAM^+$/ VE-cadherin$^+$ endothelial cells. During this process, nascent blood vessels are formed with well-defined lumens and junctional contacts characteristic of epithelia.

Numerous studies have established that *in vivo* vasculogenesis is dependent upon signal transduction involving VEGF and its receptors, Flt1 and Flk1[15-19]. For example, mice deficient in the expression of VEGF or Flk1 fail to form blood vessels. A similar outcome has been observed when early stage embryos were treated with a VEGF antagonist, sFlt1[14]. Further, augmentation of VEGF levels by introduction of exogenous VEGF has been shown to promote vasculogenesis leading to the formation of abnormally large vessels and vascular sinuses[3,14]. Here we show that addition of VEGF to 7.5 dpc allantois explant cultures also leads to transformation of small caliber vessels into sinusoidal vessels. Furthermore, addition of sFlt1 or anti-VEGF IgG to 7.5 dpc allantois explant cultures causes a failure in blood vessel formation. Taken together, we conclude that vasculogenesis occurring in the allantois explant culture system is also dependent VEGF/VEGF receptor signaling.

Unregulated VEGF signaling *in vivo* leads to hypervasculofusion, an exaggeration of the normal vascular fusion process characterized by the formation of inappropriately positioned sinuses[14]. VEGF-mediated hypervasculofusion stands as an impediment to the envisioned uses of VEGF as a therapeutic

agent[20]. Here we demonstrate that VEGF elicits hypervasculofusion in the allantois explant culture system. This represents the first *in vitro* model for this phenomenon and should aid in study of the molecular basis for the process.

We found that treatment of 7.5 dpc allantois explants with sFlt1 or VEGF antibody abrogated *in vitro* vascular network formation whereas treatment of 8.5 dpc allantois explants caused no overt effects. These results are consistent with previous findings showing that sFlt1 has differential affects on *in vivo* blood vessel formation[21]. In those studies, sFlt1 treatment disrupted the formation of the developing dorsal aortae, but did not appear to affect pre-existing blood vessels lateral to the aortae. The differential vascular outcomes observed in the *in vitro* and *in vivo* systems may reflect heterogeneity in the response of angioblasts versus endothelial cells to alterations in endogenous VEGF levels produced by sFlt1 treatment. Indeed, the ability of sFlt1 to disrupt aortic morphogenesis coincides with a developmental period when the aorta is being formed from angioblasts. In contrast, the inability of sFlt1 to affect vessels lateral to the aortae may reflect the fact that in these regions vascular morphogenesis has progressed to the point that endothelial cells are the predominant population. The basis for the apparent differential responses of angioblasts and endothelial cells may be in their relative expression of VEGF receptors. Additionally, this may reflect fundamental differences in the response of isolated cells (i.e., angioblasts) versus cells of an epithelium (i.e., endothelial cells) to VEGF. Isolated angioblasts may be more dependent on VEGF for survival than cells of an epithelium. While studies have shown that cultured endothelial cells are dependent on VEGF for survival[22], our *in vivo* and *in vitro* studies indicate that endothelial cells within intact blood vessels do not have this requirement.

Considering that vasculogenesis is now recognized as a component of adult neovascular processes[1-7], the targeting of drugs specifically to vasculogenesis represents a new direction for drug development. To date the known effectors of neovascularization have solely been screened using angiogenesis-based assays.

The allantois explant culture system provides a means to evaluate these and other agents for the ability to enhance or inhibit vasculogenesis.

Experimental Protocols

Antibodies and other proteins:

Rabbit anti-mouse TAL1 was obtained from Dr. Steven Brandt (Vanderbilt University and VA Medical Center, Nashville, TN). Rat anti-mouse- PECAM (CD31), -VE-cadherin (CD144) and -Flk1 (VEGFR-2) were purchased from BD Pharmingen (San Diego, CA). Rat anti-mouse CD34 was purchased from Research Diagnostics, Inc. (Flanders, NJ). Donkey anti-rabbit and anti-rat IgG conjugated to either fluorescein isothiocyanate or indodicarbocyanine were purchased from Jackson ImmunoResearch Labs, Inc. (West Grove, PA). Recombinant human $VEGF_{165}$, sFlt1 and goat anti-$VEGF_{165}$ IgG were obtained from R&D Systems (Minneapolis, MN).

Isolation and culture of murine allantoides:

Embryos were dissected from mice and placed into Dulbecco s PBS (DPBS) (4°C). Allantoides were then dissected away from embryos in DPBS (4°C) transferred to wells of a 4-well chamber slides (Nalgene Nunc, Naperville, IL) containing 0.4 ml DMEM (Gibco BRL/Life Technologies, Baltimore, MD), 10% FBS (Gibco), 1% penicillin, streptomycin/L-glutamine (Gibco) alone or with indicated agonists/antagonists. The extent of dilution of the culture medium with DPBS did not exceed 0.5%. Allantoides were then cultured for 24 h at 37°C, 5% CO_2 during which time they attached and flattened onto the tissue culture plastic.

Confocal microscopic analysis of allantoides cultures:

The culture medium was infused with 0.6ml of 3% paraformaldehyde, to achieve a final paraformaldehyde concentration of 2%. Fixation was allowed to proceed for 15 min, 25°C. The fixative was removed and the cultures washed twice in DPBS, 0.01% sodium azide (DPBSA). Allantois cultures were then treated with 0.02% Triton X-100,

DPBS, 0.01% sodium azide for 40 minutes to permeabilize the cells. After a 40 minute blocking treatment with 3% BSA, DPBSA, primary antibodies, diluted to 10μg/ml in DPBSA, were added and incubated for 1 h, 25°C. The cultures were washed with DPBSA and fluorochrome-conjugated anti-IgG (Jackson ImmunoResearch Labs, Inc.) added at 10μg/ml and incubated for 1 h. Following washing with DPBSA and mounted allantois cultures were analyzed using a BioRad MRC-1024 laser scanning confocal microscope (LSCM) (Bio-Rad, Cambridge, MA).

Morphometric analysis

Vascular complexity, defined as the average number of nodes and branch points within a vascular field, was determined by counting the total number of nodes and branch points within five 200μm^2 boxes placed randomly in each LSCM image. VD, defined as the percentage of a standardized optical field (1550μm^2) that is occupied by PECAM-positive cells/vessels, was determined from LSCM images using NIH Image 1.62 (white pixels representing immunolabeled vessels and black pixels avascular areas). Microsoft Excel™ was used to perform two-way Student s t-Tests (equal variances assumed) on the data.

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Table 1. Mean vascular density

	Control	VEGF	sFlt1	anti-VEGF IgG
7.5 dpc	7.4 ± 2.5 (n=16)	22.7 ± 6.0 (n=6)	2.0 ± 0.6 (n=7)	1.7 ± 0.6 (n=3)
8.5 dpc	14.0 ± 3.1 (n=20)	24.8±1.3 (n=5)	12.1 ± 3.4 (n=8)	14.8±2.2 (n=4)

*p values are >0.2, all other p values are $<1.2 \times 10^{-3}$

Figure Legends

Figure 1. PECAM immunolabeling of murine allantoides reveals the vasculature at 7.5 and 8.5 dpc. Shown are LSCM images of 7.5 dpc (A) and 8.5 dpc (B) allantoides immunolabeled with PECAM antibody. Dashed line in A outlines the border of the 7.5 dpc allantois. Bar equals 25μm.

Figure 2. Angioblasts are present in pre- and post vascularized allantoic mesoderm. A-F show LSCM images of 7.5 dpc allantoic mesoderm stained with TAL1 (A and D), Flk1 (B) and PECAM (E and F). C and F represent composites of TAL1-Flk1 and TAL1-PECAM stainings, respectively (Bar equals 10μm). G and H show LSCM images of an 8.5 dpc allantois double-labeled with antibodies to TAL1 (G) and PECAM (H). Bar equals 50μm.

Figure 3. Blood vessels formed in allantois explant cultures express endothelial-specific markers. Pre-vascularized 7.5 dpc allantois explants were cultured for 24 h and examined by LSCM using Flk1 (A and B), CD34 (C) and VE-cadherin antibodies (D). B shows a higher magnification image of isolated $Flk1^+$ cells (arrows) from the boxed area shown in A. Bars in A, C, and D equal 100μm; Bar in B equals 50μm.

Figure 4. Blood vessels generated in allantois explant cultures have lumens and form tight junctions. Pre-vascularized 7.5 dpc allantoides were cultured for 24 h and examined by LSCM using PECAM antibodies (A), light microscopy (B) and TEM (C). B shows a cross section of a vessel in an epon-embedded cultured allantois explant (1 m thick section). TEM analysis (C) reveals zonula adherens junctions (arrows) in apical aspects of endothelial cells, adjacent to the lumen of a vessel (asterisk, lumen). An arrowhead in C points to a microvillus extending from the apical surface of an endothelial cell. Bar equals 50μm.

Figure 5. Blood vessels formed in 8.5 dpc allantois explant cultures are more complex than those formed in 7.5 dpc allantois explants. Shown are LSCM images

of 7.5 dpc (A) and 8.5 dpc (B) allantoides cultured for 24 h and immunolabeled with anti-PECAM IgG. Bars equal 100μm.

Figure 6. *In vivo* and *in vitro* vasculogenesis respond similarly to modulation of VEGF signaling. A-D show QH1-stained blood vessels from lateral regions of stage 10 quail embryos, 7 h after microinjection with 25 nl of control saline (A), $VEGF_{165}$ (1μg/ml) (B), sFlt1 (1μg/ml) (C) or $VEGF_{165}$ antibody (50μg/ml) (D). Shown in E-H are PECAM-stained blood vessels formed from 7.5 dpc allantois explants cultured for 24 h in the absence of exogenous agent (E) or in the presence of $VEGF_{165}$ (1μg/ml) (F), sFlt1 (4μg/ml) (G) or $VEGF_{165}$ antibody (50μg/ml) (H). Shown in I-L are PECAM-stained blood vessels formed from 8.5 dpc allantois explants cultured for 24 h in the absence of exogenous agent (I) or in the presence of $VEGF_{165}$ (1μg/ml) (J), sFlt1 (4μg/ml) (K) or $VEGF_{165}$ antibody (50μg/ml) (L). M shows a thin section micrograph of 8.5 dpc allantois explant cultured 24 h in the presence of VEGF. Bars equal 100μm.

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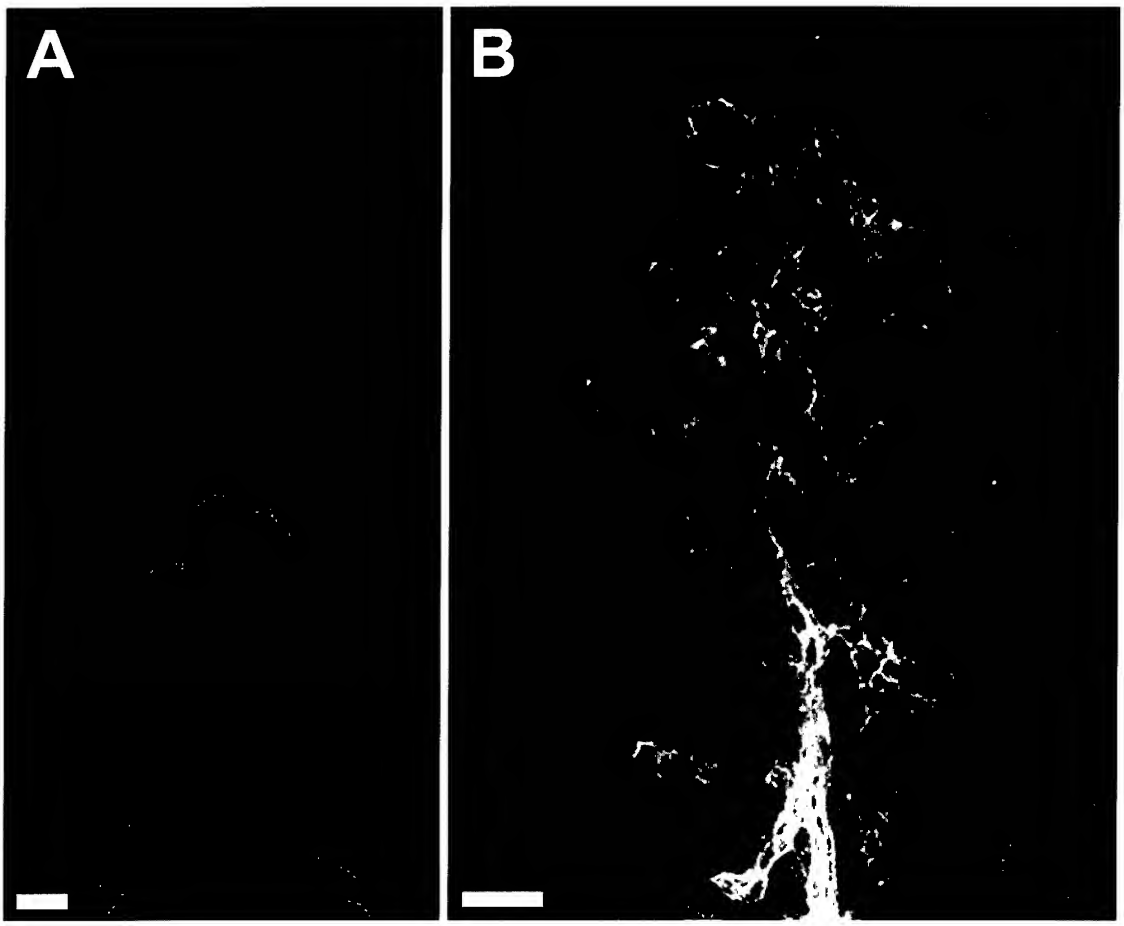

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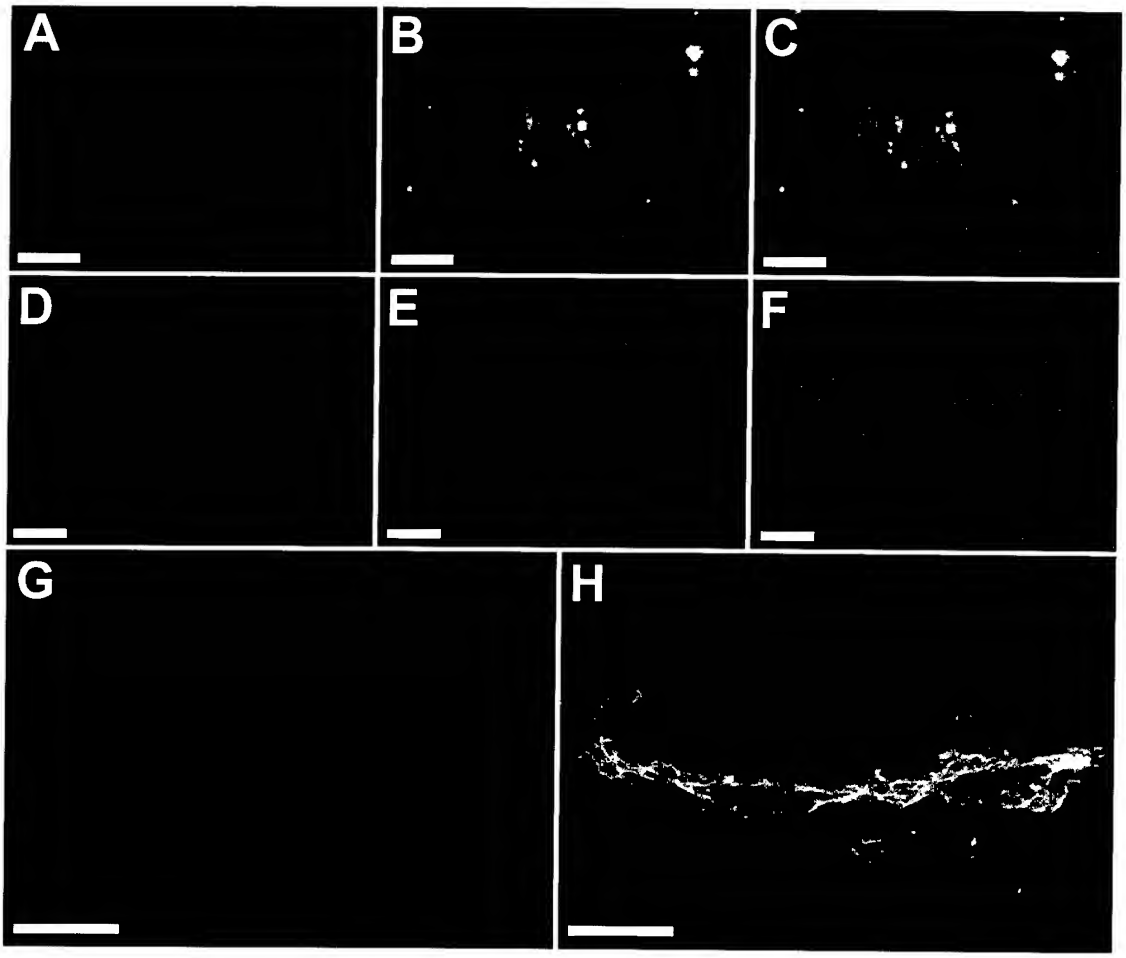
Figure 1



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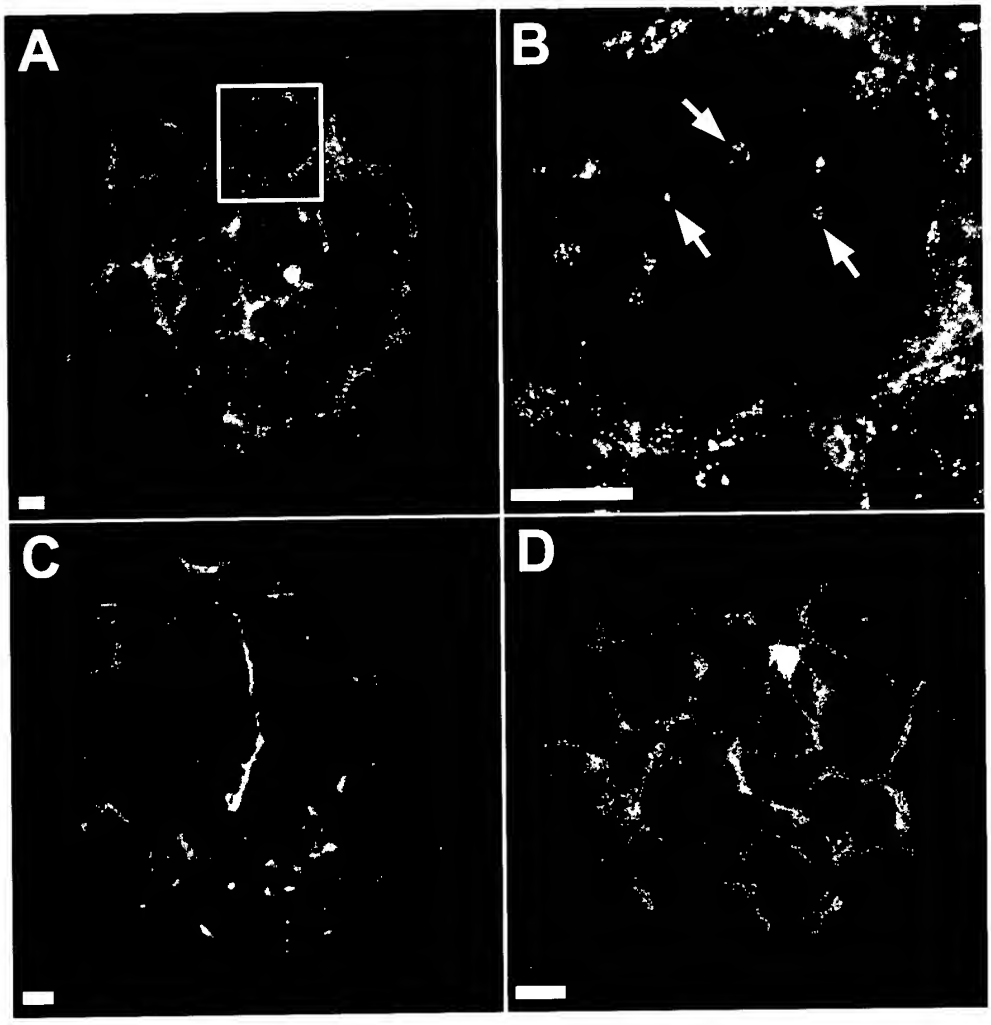

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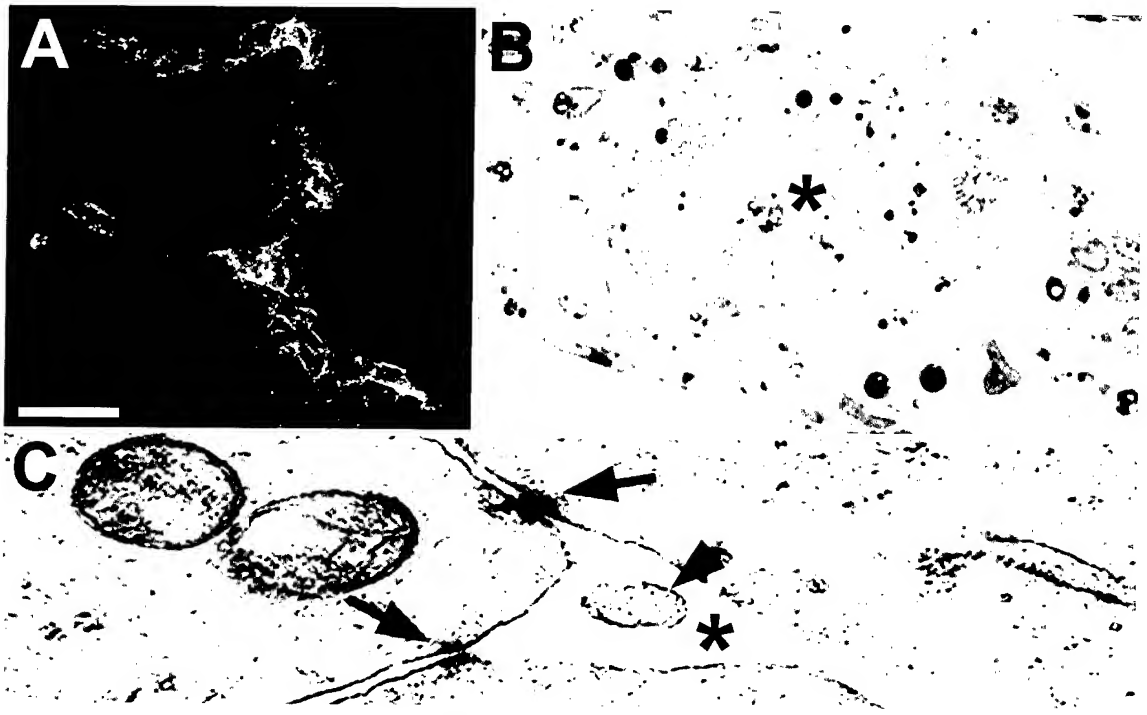

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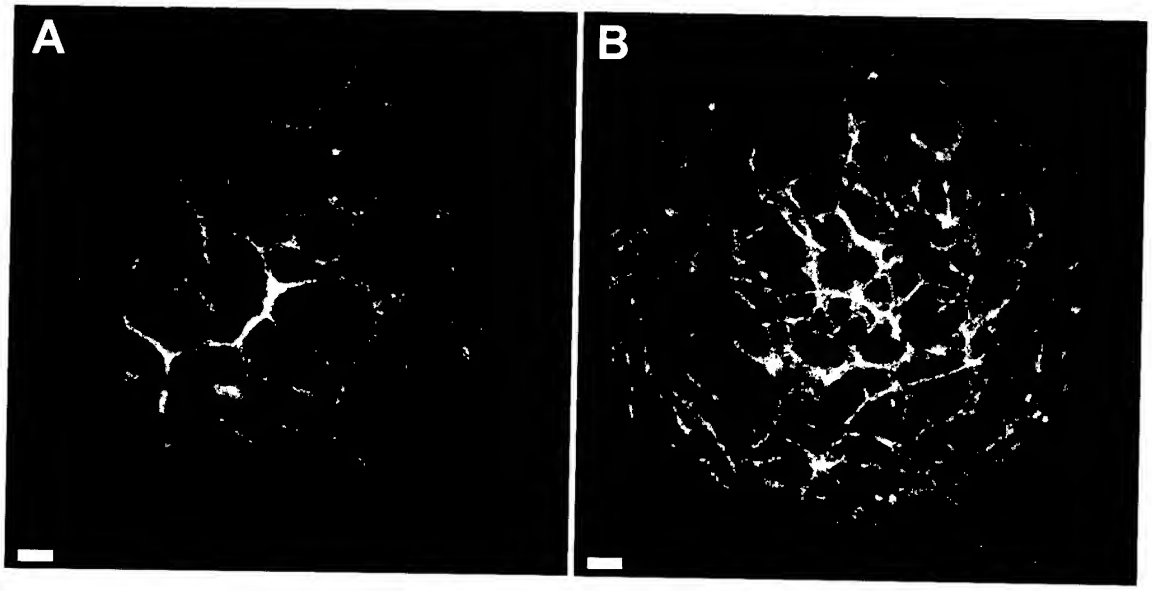

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Figure 5

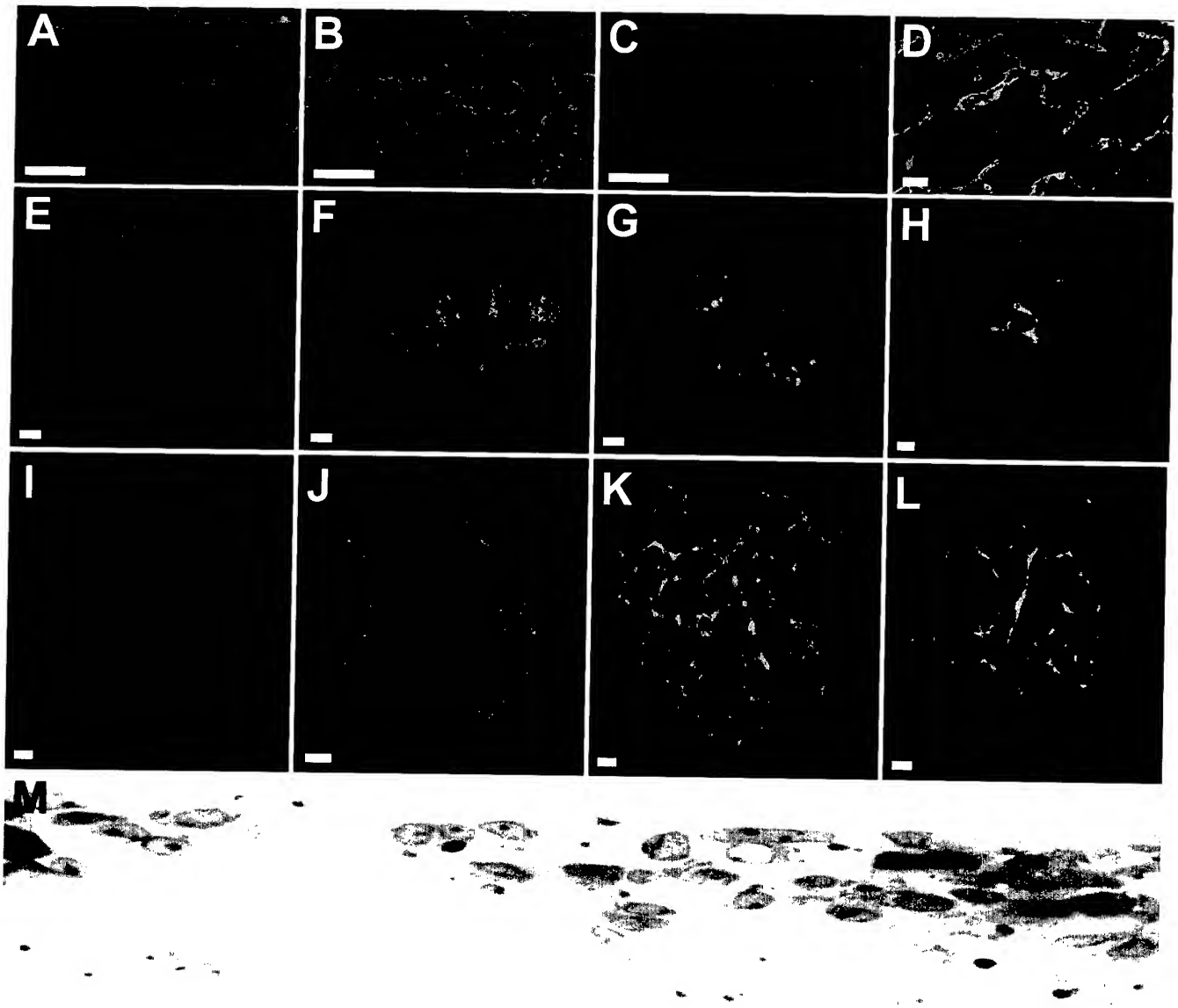

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Figure 6